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Improved CMCase production by *Spicellum roseum*

Robert W. Silman, Terry C. Nelsen, John E. McGhee and Rodney J. Bothast

Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture [1], *1815 N. University Street, Peoria, Illinois 61604, U.S.A.*

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1. SUMMARY

CMCase enzyme production by *Spicellum roseum* NRRL 13104 was increased from 2.1 to 3.4 mg reducing sugar (RS) $h^{-1} \cdot ml^{-1}$ broth in four experimental stages: (1) selection of important types of ingredients, (2) confirmation of their importance, (3) comparison of sources of the ingredient and (4) partial evaluation of concentrations.

2. INTRODUCTION

CMCase production by various isolates of *Spicellum roseum* cultured in both liquid and solid substrate was reported in previous work [1]. Additional studies to improve the process by changing media components and concentrations is reported in this paper.

Correspondence to: Robert W. Silman, Northern Regional Research Center, 1815 North University Street, Peoria, IL 61604, U.S.A.

[1] The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

3. MATERIALS AND METHODS

Spicellum roseum NRRL 13104 was maintained on potato dextrose agar (PDA), malt extract agar, malt-yeast extract agar (MYA) [2], or hay infusion agar [3]. This culture has not undergone any mutation or selection program since isolation by Bothast et al. [4]. Spore suspensions were made from slants by adding 12 ml of sterile 0.01% Triton followed by scraping and shaking. Inocula and production media were those described by Gallo [5]. Carbohydrate and nitrogen ingredients were sterilized separately. Cultures were incubated at 30 °C and shaken at 200 rpm.

Four sets of shake flask experiments were run sequentially in a scheme designed to minimize the improvement scheme. Fermentation broth samples were removed aseptically after seven days, centrifuged, and the supernatants assayed for CMCase according to the procedure described by Silman et al. [1]. Reducing sugars were determined by the potassium ferricyanide method of Hoffman [6] on an automatic analyzer (Technicon Instruments Corp.). Supernatant protein was determined by the Comassie Blue technique with reagents purchased from BIO-RAD (Bovine Serum Albumin was the standard).

Fractional factorial designs as described by Greasham and Inamine [7] were used to test for effects of the different ingredients.

4. RESULTS AND DISCUSSION

Spicellum roseum NRRL 13104 maintained on PDA agar slants produced a CMCase activity of 2.1 mg reducing sugar (RS) $h^{-1} \cdot ml^{-1}$ on Gallo's production medium when a 10% inoculum from Gallo's inoculum medium was used. However, subsequent experiments have shown that maintenance on MY agar slants and 20% inoculum is best for CMCase production. Consequently, maintenance on MY slants and 20% inoculum was standardized throughout this study designed to optimize media components for CMCase production by *Spicellum roseum* NRRL 13104. Four sequential experimental stages were followed: (1) select ingredient; (2) confirm important ingredients; (3) vary source of cellulose, protein, and mixtures of carbohydrate; and (4) vary concentration of important ingredient.

The effects of including 15 different media ingredients were tested in a replicated Plackett-Burman [7] design for 15 variables in 16 treatments (total = 32 flasks). Four ingredients (glucose, proteose, peptone, $(NH_4)_2HPO_4$ and KH_2PO_4) were found to have an effect ($P < 0.05$) on enzyme activity (Table 1). The design was 'collapsed' to the four significant ingredients and all 2- and 3-way interactions were tested and found to be unimportant ($P > 0.10$).

To further explore and refine the choice of media ingredients, a second experiment (Table 2) was run in a replicated Plackett-Burman [7] design for 7 variables in 8 treatments (16 flasks total) in which cellulose, glucose, KH_2PO_4 and proteose peptone were included in all media. KH_2PO_4 was increased from 1.5 to 1.78% to keep PO_4 level constant and $(NH_4)_2SO_4$ was used at constant NH_4^+ level to separate effects of NH_4^+ and PO_4. Also, Ca^{2+} and Mg^{2+} were combined as were Mandels' trace minerals (Zn, Fe, Mn, Co) [8]. The presence of urea and $NaNO_3$ increased enzyme activity ($P < 0.05$) and the amount of supernatant protein ($P < 0.10$). Biotin tended to increase ($P < 0.20$) enzyme activity and Mandels' salts tended to increase ($P < 0.20$) supernatant protein. The initial design allowed a collapse of the analysis on urea, $NaNO_3$ and Mandels' trace minerals into a full factorial where interactions were examined.

Table 1

Difference in enzyme activity due to inclusion of 15 different ingredients to the media

Ingredient	Amount of ingredient (g/l)	Activity without ingredient	Effect [a] of ingredient
Glucose	10.0	0.170	0.224 **
KH_2PO_4	15.0	0.209	0.146 *
$(NH_4)_2HPO_4$	2.8	0.214	0.135 *
Proteose peptone	1.0	0.219	0.126 *
Urea	0.6	0.237	0.090 +
$NaNO_3$	1.0	0.245	0.074
Biotin	0.005	0.260	0.044
Cellulose [b]	20.0	0.266	0.032
$CoCl_2$	0.002	0.271	0.024
$FeSO_47H_2O$	0.005	0.272	0.020
$ZnSO_4$	0.0014	0.274	0.015
$CaCl_22H_2O$	0.3	0.283	−0.002
$MgSO_47H_2O$	0.3	0.286	−0.008
Tween 80	1.0	0.286	−0.008
$MnSO_4H_2O$	0.0016	0.293	−0.022

[a] Effect measured as the mean of 16 flasks containing the ingredient minus the mean of 16 flasks (shown in the previous column) without the ingredient.

[b] Cellulose treatment was 10 g/l vs 20 g/l rather than absence vs presence. ** $P < 0.01$, * $P < 0.05$, + $P < 0.10$ = probability of a zero effect.

Table 2

Differences in enzyme activity and supernatant protein due to inclusion of 7 different ingredients to the media

Ingredient	CMCase (mg RS $h^{-1} \cdot ml^{-1}$)		Supernatant protein $\mu g \cdot ml^{-1}$	
	Without ingredient	Effect of ingredient [a]	Without ingredient	Effect of ingredient [a]
Urea	1.29	0.96 *	111	97 +
$NaNO_3$	1.30	0.85 *	119	82 +
Biotin	1.47	0.50 ++	151	17 ++
Mandels' T.M.	1.57	0.31	137	46
Co and Mg	1.68	0.08	152	16
Tween 80	1.70	0.04	163	−5
$(NH_4)_2SO_4$	1.86	−0.27	168	−16

[a] Effect measured as the difference between the mean of the 8 flasks containing the ingredient and the mean of the 8 flasks (shown in the previous column) without the ingredient.

* $P < 0.05$, + $P < 0.10$, ++ $P < 0.20$ for probability of a zero effect.

Table 3

Interaction between $NaNO_3$ and urea on enzyme activity and supernatant proteins

	Without urea		With urea	
	Without $NaNO_3$	With $NaNO_3$	Without $NaNO_3$	With $NaNO_3$
CMCase activity (mg RS $h^{-1} \cdot ml^{-1}$)	0.5	2.1	2.1	2.2
Supernatant protein ($\mu g \cdot ml^{-1}$)	43	181	196	222

The urea by $NaNO_3$ interaction was significant ($P < 0.05$) for both enzyme activity and supernatant protein (Table 3). When either urea or $NaNO_3$ was present in the media, the enzyme activity and supernatant protein increased by more than 4 times compared to the absence of urea and $NaNO_3$. Including both urea and $NaNO_3$ together, however, increased enzyme activity and protein were only slightly over the levels obtained when either was present by itself.

Also, an experiment on the effect of withdrawal of the 7 ingredients was run in a duplicate design

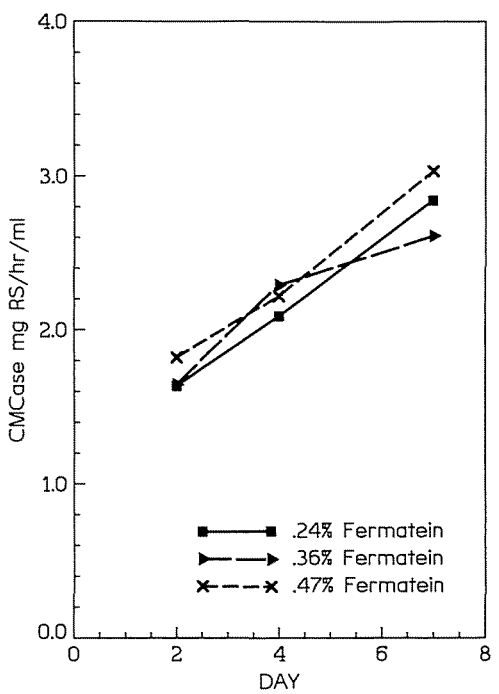

Fig. 1. Effect of Fermatein on CMCase production.

Table 4

Enzyme activity and supernatant protein after exclusion of each of 7 ingredients

Excluded ingredient	CMCase activity mg RS $h^{-1} \cdot ml^{-1}$	Supernatant protein $\mu g \cdot ml^{-1}$
Tween 80	2.56 [c]	250 [c,d]
MG salts	2.48 [b,c]	273 [d]
Trace minerals	2.32 [b,c]	189 [b]
$(NH_4)SO_4$	2.36 [b,c]	230 [c]
Biotin	2.32 [b,c]	241 [c]
$NaNO_3$	2.21 [b]	240 [c]
Urea	1.35 [a]	129 [a]
Control	2.26 [b]	245 [c,d]

[a-d] Means with the same letter are not significantly ($P < 0.05$) different as determined by Duncan's MRT.

of 8 treatments. The treatments were the 7 individual ingredients, each excluded from a combination of the other 6 ingredients, plus a control with all seven ingredients. The exclusion of urea caused a lower ($P < 0.05$) enzyme activity and less ($P < 0.05$) supernatant protein than any of the other treatments (Table 4). The exclusion of Tween 80 resulted in an enzyme activity higher ($P < 0.05$) than the control. The only treatment which caused more ($P < 0.05$) supernatant protein than the control was the exclusion of the MG salts.

Consequently, the media should include cellulose, KH_2PO_4, glucose and proteose peptone along with 0.06% urea, 0.1% $NaNO_3$, 0.0005 g/l biotin and 1 ml/l Mandels' salts.

The next step was to determine the proper pH. Mixtures of KH_2PO_4 and K_2HPO_4 were used to maintain 0.131 M buffer over a pH range of 4.6 to 9.3. A pH of 6.6 was selected based on the responses of 2.0, 3.5, 3.4, 0.7, 0.7, and 1.1 mg RS $h^{-1} \cdot ml^{-1}$ of CMCase activity and 250, 150, 310, 180, 150 and 100 g protein/ml for pH 4.6, 5.8, 6.6, 7.6, 8.1, and 9.3, respectively.

A series of flasks was then prepared to determine which sources of cellulose, organic nitrogen and supplemental saccharide to use. Based on the responses of these single flasks (Table 5), we decided to use Avicell, Fermatein and xylose for cellulose, nitrogen and saccharide, respectively.

Different concentrations of the Avicell, xylose and Fermatein were tested at days 2, 4 and 7 of

Table 5

Single flask appraisals of sources of cellulose, nitrogen and saccharide

Entity appraised	Source	CMCase activity mg RS $h^{-1} \cdot ml^{-1}$	S-N protein $\mu g \cdot ml^{-1}$
Cellulose (2%)	Ground cotton	1.5	140
	Solka-floc	1.8	180
	CMC	2.1	190
	Whatman CC31	2.6	270
	Avicell	3.4	340
Nitrogen	0.34 Corn Steep Liquor	1.8	160
	0.10 Neo-peptone	1.8	190
	0.90 Peptone	2.2	210
	0.10 Proteose peptone	2.4	250
	0.11 Tryptone	2.7	260
	0.104 Tryptose	2.7	260
	0.11 Casein hydrolysate	2.7	270
	0.19 Staley 4S	2.8	270
	0.24 Fermatein	3.2	480
Saccharide (1%)	Lactose	1.7/0.7 [a]	150/130 [a]
	Glucose	2.7/0.8	250/140
	Sucrose	2.7/0.6	290/130
	Cellobiose	3.0/1.6	250/90
	Xylose	3.4/1.1	310/130
	Xylan	3.8/0.7	340/100
	None	3.3/–	260/–

[a] Measurements from flasks which contained 2% Whatman CC31/contained no cellulose.

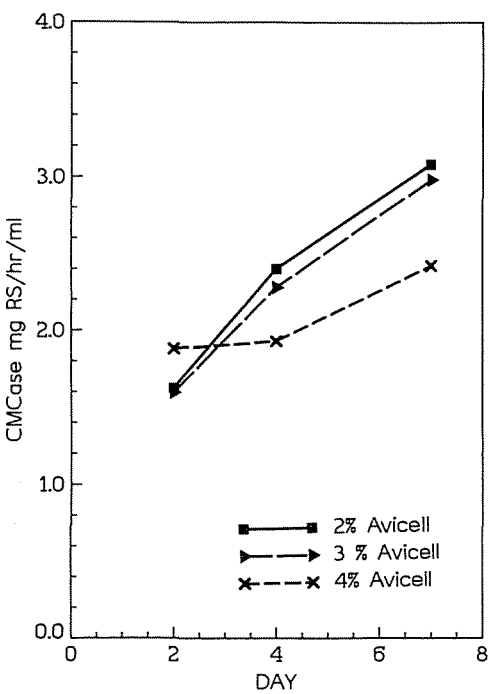

Fig. 2. Effect of avicell on CMCase production.

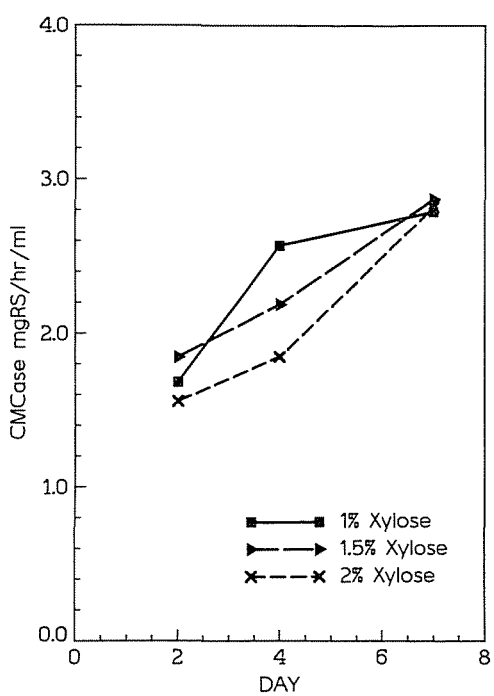

Fig. 3. Effect of xylose on CMCase production.

incubation in a Box-Behnken response surface design for four independent variables [9]. Figs. 1–3 show the CMCase results for 2, 4, and 7 days of incubation. The data points plotted are averages of at least five values. Statistical analyses showed that about two thirds of the variability was accounted for by the single effects of the three tested ingredients, while undefined interactions accounted for one third. The plots show that 0.47% Fermatein with either 2 or 3% Avicell and with either 1 or 1.5% xylose would give maximum CMCase production.

CMCase production for the similar culture conditions and ingredients did not replicate well and is probably related to the number and frequency of culture transfers. Nevertheless, in spite of the culture maintenance problem, an increase in CMCase production from ca 2.1 to ca 3.4 mg RS $h^{-1} \cdot ml^{-1}$ (62%) was achieved by (1) changing slant maintenance medium from PD to MY agar, (2) increasing inoculum from 10 to 20%, and (3) improving media components and concentrations. Improved CMCase production was achieved with relatively few experiments.

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